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<p>13. Abstract (Maximum 200 Words) <i>(abstract should contain no proprietary or confidential information)</i></p> <p>The objective of this project was evaluate the Peruvian medicinal plant in the form of crude extracts and/or as pure compounds, for the ability to inhibit breast cancer growth and prevent mammary gland transformation. While there is anecdotal information that suggests that this plant may be an effective cancer treatment, there is little scientific evidence supporting such claims. Therefore, it is important to investigate potential biological effects. We have found that very low doses of an aqueous extract can significantly inhibit the growth of a number of breast cancer cell lines. The median effective dose (ED50) in culture was approximately 10 µg/ml. All of the breast cancer cell lines that were growth inhibited by the extract overexpress the <i>erbB-2</i> (HER-2/neu) tyrosine kinase receptor. In contrast, no significant growth inhibition was observed in breast cancer cell lines expressing low levels of this receptor. The extract inhibited <i>erbB-2</i> autophosphorylation in breast cancer cell lysates, suggesting that one or more compounds in the extract may inhibit cell signaling. We also showed that the extract has cancer chemopreventive activity, as demonstrated by its ability to inhibit formation of chemically induced preneoplastic lesions in a mouse mammary gland organ culture model. These effects were measured as part of a rigorous scientific examination into the mechanisms of the activities mediated by the plant. Although our results in vitro and in vivo are quite encouraging, it will be quite premature and irresponsible to promote its use as an anticancer therapy. We anticipate that specific agents in the plant will inhibit breast cancer cell growth through chemotherapeutic and/or chemopreventive effects and that compounds isolated from the plant are potential candidates in treating breast carcinomas.</p>				
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INTRODUCTION

Worldwide, cancer accounts for more than 6 million deaths annually (1). According to estimates from the American Cancer Society (2), approximately 1.2 million new cancer cases were to be diagnosed in 1998 in the United States, including 178,700 diagnoses of breast cancer in women. Polypeptide growth factors play important roles in both normal and pathological development of the breast. Such growth factors promote cell proliferation, motility and invasiveness of epithelial cells *in vitro*, properties that are required for tumor invasiveness and metastasis. Recent experimental evidence suggests that estrogens stimulate breast proliferation in hormone-dependent cells by upregulation of an autocrine stimulatory loop involving epidermal growth factor receptor related tyrosine kinases (*erbB-2*) (3,4). The *erbB-2* oncogene product has been shown to be expressed in approximately 25-30% of breast cancer patients, and has been correlated with poor prognosis and unfavorable survival rate (5,6). Frequently, patients whose tumors express *erbB-2* receptors do not respond well to conventional therapies, emphasizing the need for more aggressive therapies. Although the exact role of *erbB-2* in tumor development has yet to be elucidated, tyrosine kinase-mediated signal transduction appears to play an important role in breast cancer progression and metastasis.

Although there are currently over 50 clinically approved anticancer drugs available, no curative treatment exists for advanced metastatic breast cancer. Commonly used hormonal and chemotherapeutic agents can, in some cases, lead to transient regression of tumors, and can palliate symptoms related to cancer. However, these treatments are often accompanied by side effects and eventually become ineffective in controlling cancer and its symptoms. Newer strategies for chemotherapeutic drug development include high-dose chemotherapy with hematopoietic stem cell support, and the selective targeting of agents to molecular and cellular abnormalities. One such strategy has been the generation of tyrosine kinase inhibitors. Inhibitors of tyrosine kinases have been shown to produce a wide variety of potential antitumor effects, including blocking of cell proliferation, and induction of differentiation or apoptosis (7-9).

Natural products have proven to be a valuable source of novel, highly effective drugs. Presently, more than 120 therapeutic agents dispensed by pharmacies originate from plants (10), many of which are still obtained directly from the plants in which they are synthesized (11). Several clinically useful anticancer drugs, including paclitaxel (Taxol) and vinblastine (Velban), are plant-derived. The first tyrosine kinase inhibitor discovered, quercetin, occurs in plants (12). Since then, many other naturally occurring tyrosine kinase inhibitors have been found. However, most natural tyrosine kinase inhibitors are not specific to one particular enzyme. Many naturally occurring molecules have served as the structural basis for the development of synthetic inhibitors, or tyrphostins (13). With a large number of known plants remaining to be analyzed, in addition to the multitude of species not yet discovered, it seems likely that more plant extracts and plant-derived compounds which demonstrate more specificity to tyrosine kinases will be discovered.

Chemoprevention is the systematic use of non-cytotoxic nutrients or pharmacological agents to inhibit, delay, or reverse the process of carcinogenesis, ultimately providing benefit to public health by lowering the incidence of human cancer (14,15). Since most cancers develop through a cascade of multiple events over a long period, there is a window of opportunity of up to 20 years or more during which active measures may be taken in an effort to interrupt the carcinogenic process. The identification of intermediate biomarkers, such as premalignant lesions, that can be used as surrogate end-points for cancer has allowed for the design of relatively short-term clinical studies (16) that are useful for studying biological activity, and for the selection of agents for long-term clinical trials (17) of chemopreventive drugs.

More than 500 compounds belonging to over 20 classes of chemicals have demonstrated some type of chemopreventive activity (18,19). Many of these compounds occur naturally in edible plants or in plants with traditional medical uses, thus they may be consumed on a regular basis in large quantities. So far, no widely accepted system for classifying chemopreventive agents exists. The most common way to classify chemopreventive agents is based on the multistep model of carcinogenesis, with inhibition of initiation, promotion and progression being the three main categories for mechanism of action (20). This classification system can be expanded to include inhibition of invasion and metastases (21) as well.

Inhibitors of initiation can be classified by at least 23 different mechanisms of action (21). Examples include inhibition of promutagen/procarcinogen activation, induction of detoxification mechanisms, reaction with active oxygen species, and modulation of DNA repair systems. Free radical scavenging, inhibition of cell proliferation, induction of cell differentiation, and modulation of signal transduction are considered potential mechanisms for the inhibition of tumor promotion (21). Modulation of signal transduction, effects on growth factors, effects on hormones, effects on the immune system and inhibition of neovascularization are potential mechanisms for inhibition of tumor progression (21). Many chemopreventive agents have multiple actions, making it difficult to assign a specific mechanism, but potentially enhancing the chemopreventive effects of agents through multiple inhibitory effects. For example, tamoxifen, an antiestrogen in breast tissue, demonstrates several other activities that could be related to its chemopreventive activity, including inhibition of protein kinase C (22), of ornithine decarboxylase, and of other enzymes involved in carcinogenesis (15).

Uncaria tomentosa (UT) is a woody vine native to the Peruvian rain forest. It is commonly referred to in Spanish as *uña de gato*, or "cat's claw". A survey of the NAPRALERT database (Natural Products ALERT) indicates that preparations from the root bark of UT are used ethnomedically (traditionally) as an anti-inflammatory, and for the treatment of arthritis, intestinal disorders and cancer. Such extensive traditional use without reported toxicities has led to the widespread commercial availability of this plant. However, there is minimal clinical evidence that proves safety or even any efficacy in humans. One report does indicate a lack of toxicity in a variety of *in vitro* assays (23). Biological activities attributed to extracts of UT include anti-mutagenicity, anti-inflammatory, antioxidant, phagocytosis stimulation, and induction of apoptosis (24-28). However, there are not extensive data in the literature supporting any specific therapeutic use. Several compounds have been isolated from this plant, primarily oxindole alkaloids and

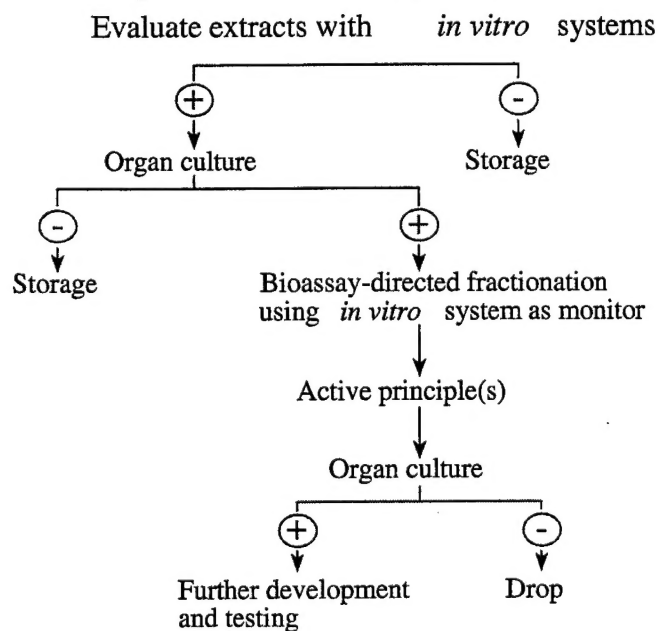
triterpenoids. Antiviral activities have been reported for triterpene quinovic acid glycosides (29). Oxindole alkaloids have demonstrated anti-inflammatory and immuno-stimulating effects (27). In addition, the oxindole alkaloids have demonstrated growth inhibitory effects in human leukemia cell lines (30). Recently, synthetic oxindole alkaloids were shown to inhibit bFGF tyrosine kinase activity (31).

We have recently found that UT inhibited the growth of several *erbB-2* overexpressing breast cancer cell lines, while having a markedly lower effect on low or non-expressing *erbB-2* cell lines. The extract suppressed *erbB-2* tyrosine kinase activation in a dose-dependent manner. Moreover, dietary administration of this extract inhibited the growth of *erbB-2* overexpressing cells in athymic nude mice. In addition, chemical transformation of normal mouse mammary glands to a preneoplastic phenotype was blocked by treatment with this extract. However, the mechanism of action of UT in both anti-tumor and chemoprevention is still unknown.

We propose to isolate the active compound(s) and to study the mechanism by which UT inhibits breast cancer growth and transformation. Identifying the mechanism will determine the potential of this plant for use in therapy for breast carcinoma, as well as for chemoprevention in high-risk populations, such as men who have undergone radical mastectomy. We will test the completely herbal extract "as used traditionally"

as well as individual constituents. Our studies are designed to elucidate the biological mechanisms by which the extract of UT, or some chemical constituent(s) contained within, inhibits the proliferation of breast cancer cells. Identifying the mechanism by which this extract inhibits breast cancer proliferation will determine the potential of this plant as a source of a new therapy for malignant breast carcinoma, as well as for other cancers that express the *erbB-2* receptor tyrosine kinase. In addition, these studies will determine whether components in an extract of the UT have greater potential as a chemotherapeutic or a chemopreventive agent, and for which populations; these results will assist in the development of clinical trials.

Experimental Approach #2



BODY AND KEY RESEARCH ACOMPLISHMENTS

1: An extract derived from UT inhibits the growth of *erbB-2* overexpressing cells. In an effort to discover naturally occurring substances that inhibit breast cancer cell growth, we tested an aqueous extract of the medicinal plant UT. As shown in Table 1, we found that low doses of the extract significantly inhibited growth of a number of cultured human breast cancer cell lines. Interestingly, all of the cell lines inhibited by the extract overexpress the *erbB-2* tyrosine kinase receptor. The dose response curve for several cell lines tested, other cell lines (data not shown) responded in a similar manner. The median effective dose (ED₅₀) for the most responsive cell lines was approximately 10 μ g/ml. In contrast, low or no significant inhibition was observed when the breast cancer cell lines that express low levels of *erbB-2* were treated with the extract. Moreover, the growth of a normal (immortalized) breast cell line was not inhibited by exposure to UT (data not shown), demonstrating a lack of nonspecific toxic response to the extract. Our results indicate that one or more compounds present in this UT inhibit specifically the growth of *erbB-2* overexpressing cancer cells.

TABLE 1. Response of different breast cancer cell lines to UT				
Cell Line	ER	EGFR	<i>erbB-2</i>	Sensitivity to UT
MCF-7	++++	+/-	+/-	-
MDA-MB-231	-	+	-	-
MDA-MB-468	-	++++	-	+/-
MDA-MB-175	+	ND	-	-
ZR75B	+	+/-	-	-
MDA-MB-157	-	ND	-	-
BT549	-	-	-	-
HS578T	-	-	-	-
MDA-MB-435	-	-	+	+/-
T47D	+++	+	++	++++
BT474	++++	+	++++	++++
MDA-MB-453	-	-	++++	++++
AU565	-	++	++++	++++
SK-BR-3	-	++	++++	++++

Cell lines in culture were treated (in triplicate) with various concentrations of extract. After 72 hours of incubation, cells were counted. Dose response curves were generated by comparisons to untreated controls. ND= Not determined

2: Dietary administration of an extract derived from UT reduces the growth of *erbB-2* overexpressing cells *in vivo*. As proof of concept and to determine the chemotherapeutic activity of UT, we initiated studies *in vivo*, using BT474 breast cancer cells, an *erbB-2* overexpressing cell line previously used to assess activity of anti-*erbB-2* agents such as Herceptin. Our studies were conducted in as tumor xenographs ovariectomized athymic nude mice (12 mice/group), supplemented with estradiol (E2). Of note,

BT474 cells overexpress *erbB-2* and it has been the model used for the development of Herceptin, a drug approved for the treatment of breast cancer tumors which overexpress the *erbB-2* receptor.

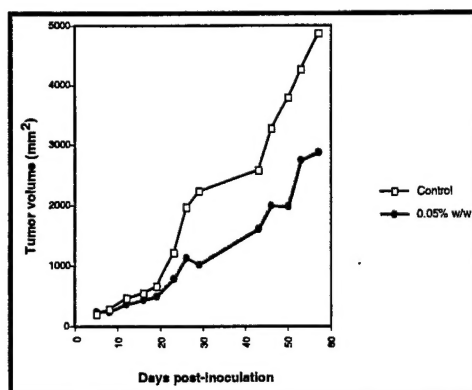


FIGURE 2: ADMINISTRATION OF AN EXTRACT OF THE UT INHIBITS TUMOR GROWTH OF *erbB-2* OVEREXPRESSING BREAST CANCER CELLS *IN VIVO*.

Female ovariectomized athymic nude mice, following inoculation with 1×10^6 breast cancer tumor cells and supplemented with estrogen, were provided a diet consisting of standard laboratory chow alone or chow containing 0.05% (w/w) of lyophilized aqueous extract. The experiment was carried out for 60 days. During the course of the experiment, mice were

examined daily to monitor health. Tumor volume was measured twice per week. Results are expressed as % inhibition relative to mice fed a control diet without the extract. The standard deviation was smaller than 15%. As shown in Figure 2, dietary administration of UT resulted in a statistically significant reduction in the growth of BT474 human breast cancer cells implanted into nude mice. It is important to note that the variability among the animals in each group was less than 15%. Our preliminary data demonstrated that when an extract of the UT was mixed with the diet, tumor formation of breast cancer cells was diminished. Additional studies demonstrated the ability of the extract to inhibit tumor formation of *erbB-2* overexpressing cells *in vivo*. Further studies need to be performed with isolated fractions to demonstrate the efficacy of UT.

3: An extract derived from the UT blocks *erbB-2* autophosphorylation. Since an extract of UT appears to preferentially inhibit the growth of *erbB-2* overexpressing cell lines both *in vitro* and *in vivo*, we tested the ability of UT to inhibit autophosphorylation of the *erbB-2* receptor. The extract demonstrated dose-dependent inhibition of *erbB-2* autophosphorylation in an immuno-complex kinase assay. Autophosphorylation is precise and is required for tyrosine phosphorylation of substrates to occur. Thus, inhibition of autophosphorylation should lead to a complete shutoff of the signaling pathway, promoting inhibition of tumor growth. It would be beneficial at this point to know the structure of the kinase inhibitor in UT because it would aid in determination of the extract's specificity for *erbB-2*, since many other natural kinase inhibitors are nonspecific. Knowing the structures of the active compound(s) would be beneficial in the standardization of commercially available extracts.

Autophosphorylation of *erbB-2* was measured in an immuno-complex tyrosine kinase assay. 96-well plates were coated with goat anti-mouse immuno-globulin, and then incubated for 6 hours with monoclonal antibody. Nonspecific binding sites were blocked with gelatin (3%) and glycine in phosphate-buffered saline. Membrane preparations of cells overexpressing the *erbB-2* receptor (MDA-MB-453) were applied ($1-2 \times 10^6$ cells/well) and incubated overnight. After washing, various concentrations of the extract (diluted in kinase assay buffer) were applied to the wells and incubated for 20 min at room temperature. Proteins

were eluted with sample buffer, subjected to SDS-PAGE, and transferred to nitrocellulose membranes. DMSO was used as control.

- Anti-phosphotyrosine immuno-blotting was performed and proteins were visualized by enhanced chemo-luminescence.
- Bands were quantified using AlphaEase software. Quantification of the kinase assay.

4: An extract from UT contains compounds that generate antioxidant activity. The extract demonstrated the ability to scavenge free radicals of 1,1-diphenyl-2-picrylhydrazyl in ethanol. This data is in agreement with previously published reports of antioxidant activity. The ability of UT to mediate an antioxidant effect suggests an anti-initiation and/or anti-proliferation mechanism of chemoprevention.

COMPOUNDS WITH ANTIOXIDANT ACTIVITY

Test samples were dissolved in DMSO and added in triplicate to 96-well plates (5 μ l/well). DPPH- (1,1-Diphenyl-2-picrylhydrazyl) was dissolved in absolute ethanol and 95 μ l aliquots were added to each test sample (final concentration, 300 μ M). The plates were covered with parafilm and incubated at 37°C for 30 min. Following incubation, the plates were shaken briefly and the absorbance A_{515} was measured with a microtiter plate reader. The absorption values obtained for each treatment were averaged, and the resulting values were expressed as a percentage of solvent-treated controls. The standard deviation was smaller than 15%.

5: An extract derived from the UT inhibits formation of preneoplastic mammary lesions. A good correlation has been established between agents capable of inhibiting preneoplastic lesions in this model and those capable of preventing mammary carcinogenesis in full-term animal studies. It is known that 7,12-dimethylbenz[a]anthracene induces specific transformation of the mammary glands (DMBA). A representative example of assay results, at a concentration of 10 μ g/ml the extract showed 75% inhibition of preneoplastic lesions induced by DMBA with an *in vitro* mouse mammary gland organ culture (MMOC) system. The data demonstrates that UT is capable of inhibiting transformation of mammary cells. Thus, one or more compounds in this extract can be developed as chemopreventive agents.

REPORTABLE OUTCOMES: We have observed the ability of a specific UT extract to inhibit the proliferation of *erbB-2* overexpressing breast cancer cell lines. Dietary supplement and/or administration of the UT extract inhibited the tumor growth of human breast cancer cells in athymic nude mice. The mechanism of action of the compounds in the UT that inhibited cell proliferation tumor growth of *erbB-2* cells, appear to be by blockage of the *erbB-2* tyrosine kinase. Additionally, we have clearly demonstrated that the UT plant contains chemopreventive compounds, as demonstrated by the ability of the extract to block DMBA induced transformation of the mammary gland and by the presence of antioxidant activity. Taken together, these data demonstrates that the extract from the UT plant contains a candidate compound(s) with *erbB-2* tyrosine kinase inhibitory properties as well as a yet unknown compound(s) that demonstrate chemopreventive activity, and supports further investigation. Thus, additional studies are

necessary to establish the identity, specificity, and the mechanism of action of the compounds present in the UT extract.

CONCLUSIONS: The biological responses we have observed in breast cancer cells treated with extracts of *Uncaria tomentosa* could not have been predicted from most activity reported in the literature, although much of the data reported does support chemotherapeutic and/or chemopreventive effects. This plant is used commonly as an herbal remedy in the United States therefore it is readily available. However, it would be quite premature and irresponsible to promote its use as an anticancer therapy based solely on ethnomedical claims and a few *in vitro* studies. Thus, it is very important to thoroughly evaluate the effect of the plant, both in the laboratory setting and in clinical trials. We anticipate that *Uncaria tomentosa*, as used traditionally as an extract, as well as selective isolated compounds will inhibit breast cancer cell growth through chemotherapeutic and/or chemopreventive effects. We anticipate that compounds isolated from UT will be potential candidates in treating a large number of malignancies, in which *erbB-2* is overexpressed. In particular for the treatment of breast carcinomas breast carcinomas in which overexpression of *erbB-2* has been correlated with poor prognosis and overall survival.

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APPENDICES

Not applicable